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(54) Title: MONOMERIC AND DIMERIC ANTIBODY-FRAGMENT FUSION PROTEINS

(57) Abstract

The present invention describes a new class of antigen binding molecules which contain Fv-fragments of an antibody but do not use the constant antibody domains. They can also dimerize with other antibody fragment molecules or with non-antibody fragment molecules to form bi- or multifunctional antibody-fragment fusion proteins and so-called miniantibodies, respectively. The new fusion proteins can be used in the broad field of diagnostic and therapeutical medicine.

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Monomeric and Dimeric Antibody-Fragment Fusion Proteins

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The present invention describes a new class of antigen bin-
ding molecules which contain Fv-fragments of an antibody but
do not use the constant antibody domains. They can also
10   dimerize with other antibody fragment molecules or with
non-antibody fragment molecules to form bi- or multifunctio-
nal antibody-fragment fusion proteins and so-called mininan-
tibodies, respectively. The new fusion proteins can be used
in the broad field of diagnostic and therapeutical medicine.

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Background of the invention

Since a few years there is a great interest in the biotechno-
logical field to modify naturally occuring antibodies in
20   order to obtain more specified and more individual antibody
species. Therefore, attempts have been made to produce (modi-
fied) antibody fragments.
All naturally occuring antibodies of all classes have at
least two binding sites. This enables them to bind to a
25   surface with a greater functional affinity (also called
avidity) than monovalent fragments, such as Fab fragments.
Over the last few years, methods have been described
(Skerra and Plückthun, 1988, Science 240, 1038-1040 ; Better
et al., 1988, Science 240, 1041-1043) with which

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functional antibody fragments can be produced in Escherichia
coli. These include the Fv fragment (the heterodimer
consisting of V_H and V_L) and the Fab fragment (con-
sisting of the complete light chain with the domains V_L
and C_L as well as the first two domains of the heavy
chains V_H and C_{H1}).

The Fv fragment, however, has a tendency to dissociate into V_H
and V_L and therefore, it is advantageous to link the two
domains covalently. One particular way of linking them is by
designing a peptide linker between them, either in the orien-
tation V_H-linker-V_L or V_L-linker-V_H (Bird et al.,1988, Science
242, 423; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85,
5879) The resulting fragments are called single-chain Fv
fragments.

All these fragments are, however, monovalent. We describe in
this invention a method to engineer small dimerization
domains based on peptides forming amphipathic helices. These
peptides will be referred to as "intercalating", but this
term is only meant to express the ability of targeted
association and not a restriction referring to a particular
structure of the dimerization interface.

While the methodology described here, is in principle appli-
cable to either Fab, Fv or scFv fragments, it is the latter
for which their use is most advantageous. In this case biva-
lent fragments can be constructed cf very small size, and
still the dissociation into V_L and V_H as well as the wrong
matching of fragment chains, e.g. V_L-V_L, can be prevented.

Antibody fragments of small size are of particular advantage
in many applications. In diagnostic applications (e. g.
ELISA, RIA, etc.), the smaller molecules surface decreases
the problems of nonspecific interactions, which are known to
5   frequently involve the constant domains. The same is true in
using antibody fragments as ligands in affinity chromatogra-
phy. In tumor diagnostics or therapy, it is important that a
significant proportion of the injected antibody penetrates
tissues and localizes to the tumor, and is dependend on the
10   molecular dimensions (Colcher et al., 1990, J. Natl. Cancer
Inst. 82, 1191-1197). Expression yields and secretion
efficiency of recombinant proteins are also a function of
chain size (Skerra & Plückthun, 1991, Protein Eng. 4, 971)
and smaller proteins are preferred for this reason. There-
15   fore, molecules of a small size are advantageous for several
reasons.

Previously, decreasing the molecular dimensions of the anti-
body meant the preparation of proteolytic fragments.
20   The smallest bivalent fragments, (Fab)'$_2$ fragments, are still
about twice the size of the present fragments of this inven-
tion. Therefore, these new fragments combine three features:
(a) small size, (b) bivalence or bifunctionality and (c)
ability of functional expression in E. coli.
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There is great interest in bifunctional antibodies in a large
number of areas. Bifunctional antibodies may be defined as
having two different specificities for either two different
antigens or for two epitopes of the same antigen.
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There are currently a number of methods how to produce bifun-
ctional antibodies. However, none of the existing methods
allows to produce exclusively bifunctional antibodies in
vivo, but rather a mixture of molecular species always occur,
5   requiring complicated and expensive separation procedures.

Four principal methods can be distinguished. In the first,
chemical crosslinking is used, which may advantageously use
heterobifunctional crosslinkers. By this method, whole anti-
10   bodies (Staerz et al., 1985, Nature 314, 628; Perez et al,
1985, Nature 316, 354-356), Fab fragments (Carter et al.,
1992, Biotechnology 10, 163) and scFv fragments (Cumber et
al., 1992, J. Immunol. 149, 120) have been chemically
crosslinked after purification.
15

The second previous method involved the fusion of two hybri-
domas to give a so-called heterohybridoma or "quadroma". In
this method, any light chain can pair with any heavy chain,
and the two heavy chains can give homodimers or heterodimers
20   resulting in very complicated product mixtures (Milstein &
Cuello, 1983, Nature 305, 537).

The third method is related to the second and consists of
transfecting two expression plasmids into a hybridoma cell,
25   encoding the heavy and light chain of the second antibody
(Lenz & Weidle, 1990, Gene 87, 213) or a retroviral vector (
De Monte et al., 1990, Acad. Sci. 87, 2941-2945). However,
once introduced, the product mixture is identical as in the
second procedure.

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Finally, antibodies have been reduced, mixed and reoxidized
(Staerz & Bevan, 1986, Immunology Today 7). Again, very
complicated product mixtures are obtained requiring sophisti-
cal separation and quality control procedures.

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Thus a method is still needed allowing the isolation of
exclusively heterodimeric antibodies directly without the
complicated preparation required from chemical crosslinking.
In the present invention, this problem is solved by (i)
10   covalently linking corresponding VH and VL domain in a scFv
fragment and (ii) using dimerization domains only allowing
the formation of heterodimers, such as certain leucine zip-
pers and derivatives.

15   Another important consideration in the present invention was
the desire to make the MW of the bispecific antibody as small
as possible for reasons explained above in detail. This was
achieved by using scFv fragments.

20   A number of uses of bispecific antibodies bave been descri-
bed, and most of them would benefit from this new technology.
For example, bispecific antibodies are of great interest in
tumor therapy. One arm of the antibody may bind to a tumor
marker, the other arm to a T-cell epitope, a toxin, or a
25   radionuclide binding peptide or protein to bring a killing
function close to the tumor cell. In diagnostics, one arm may
bind to the analyte of interest and the other to a principle
which can easily be quantified, e. g. an enzyme. Finally, in
cellular applications, it may be advantageous to obtain
30   higher selectivity in binding, if two different epitopes or
the same protein complex can be recognized or if two diffe-
rent proteins can be recognized on the same cell surface.

Thus, it was object of the invention to create new individual
and stable antibody fragment fusion proteins with bi- or even
mulitfunctional binding sites.

5   It has been found that antibody fragment fusion proteins
containing Fv-fragments could be produced by genetic enginee-
ring methods which show specified and improved properties.

Object of the invention is, therefore, a monomeric antibody-
10   fragment fusion protein essentially consisting of a Fv-frag-
ment of an antibody and a peptide which is capable to dime-
rize with another peptide by noncovalent interaction.

The term "noncovalent interaction" means every existing under
15   normal conditition stable linkage which is not related to a
covalent binding, for example linkage by Van der Waal's
forces, (steric) interdigitation of amphiphilic peptides,
especially peptide helices, or peptides bearing opposite
charges of amino acid residues. The correspondingly effective
20   peptides are called above and below interactive or intercala-
ting peptides.

The amphiphilic peptides consist of up to 50 amino acids.
Preferrably they consist of 10 to 30 amino acids. In a pre-
25   ferred embodiment of the invention the interactive peptide is
a peptide helix bundle (comprising of a helix, a turn and
another helix, see above). In another embodiment the interac-
tive peptide is a leucine zipper consisting of a peptide
having several repeating amino acids, in which every seventh
30   amino acid is a leucine residue. In other cases according to
the invention the peptide bear positively or negatively

charged residues, e.g. lysine (positively charged) or gluta-
mic acid (negatively charged) in a way that this peptide can
bind to another peptide (of a second monomeric unit) bearing
opposite charges.

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The Fv-fragment and the intercalating peptide are linked
together either directly or by a linker peptide, preferrably
by a linker peptide. In a preferred embodiment the linker
peptide is a hinge region sequence of an antibody.

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As defined, the Fv-fragment consists of the V_L and V_H region
of an antibody. The Fv-fragment according to the invention is
preferrably a single chain fragment. Single chain fragments
can be obtained by standard techniques using standard linker

15   molecules.

Furthermore, object of the invention is a dimeric fusion
protein essentially consisting of two monomeric fusion pro-
teins, wherein the linkage of the monomeric units bases on

20   noncovalent interaction of identical or different peptides,
characterized in that at least one monomeric unit is an
antibody-Fv-fragment fusion protein as defined above.

If the dimer contains two Fv-fragments, the Fv-fragments may

25   be the same (identical antigen binding sites) or may be
different (different antigen binding sites). In these cases
mono- and bispecific (Fv)- miniantibodies can be obtained.
According to the invention bispecific mini-antibodies are
preferred.

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The interactive peptides may be the same or may be different;
preferrably, they are identical. The intercalating peptides
may be associated in parallel or in antiparallel fashion.

5   Object of the invention is, therefore, above all, a dimeric
       fusion protein consisting of two Fv-fragments with different
       specificity (antigen binding sites) and identical intercala-
       ting helix peptides, the antibody fragments and the peptides
       are linked together by a hinge region sequence.
10

       Furthermore, object of the invention is a dimer consisting of
       a monomeric unit containing a Fv-fragment and another monome-
       ric unit wherein the Fv-Fragment was replaced by a non-anti-
       body peptide. The non-antibody peptide may be a toxin, like
15   ricin, a chelator- or metal binding peptide, or an enzyme
       (e.g. marker enzyme), or a peptide bearing a detectable lable
       (e.g. a radioisotope).

       The non-antibody peptide can also bear a corresponding bin-
20   ding site for said groups, binding sites directed to T-cells
       or T-cell fragments included.

       Furthermore, the invention relates to monomers and dimers, as
       defined above, wherein the interactive peptide(s) is (are)
25   additionally fused at the C-terminus to target proteins/pep-
       tides as mentioned above, the corresponding binding sites
       included. Thus, the resulting fusion proteins and miniantibo-
       dies, respectively, are multifunctional.

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The invention relates, furthermore, to a process for prepara-
tion of a monomeric antibody fusion protein as defined above,
characterized in that the genes coding for the Fv-fragment,
the interactive peptide and, if desired, the linking peptide
5   are cloned into one expression plasmid, a host cell is trans-
formed with said expression plasmid and cultivated in a
nutrient solution, and the monomeric fusion protein is
expressed in the cell or secreted into the medium.

10   Object of the invention is, finally, a process for prepara-
tion of a dimeric fusion protein as defined above, characte-
rized in that the genes coding for the complete monomeric
fusion proteins or parts of it are cloned at least into one
expression plasmid, a host cell is transformed with said
15   expression plasmid(s) and cultivated in a nutrient solution,
and either the complete dimeric fusion protein is expressed
in the cell or into the medium, or the monomeric fusion
proteins are separately expressed and the noncovalent lin-
kage between the two monomeric units is performed in the
20   medium or in vitro, and in the case that only parts of the
fusion proteins were cloned, protein engineering steps are
additionally performed according to standard techniques.

The dimeric Fv-fragments containing fusion proteins according
25   to the invention show a high avidity against corresponding
antigens and a satisfying stability. These novel bivalent or
bifunctional molecules can be prepared as folded and assem-
bled molecules in E. coli. These miniantibodies are compa-
tible with functional expression by secretion.

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Detailed description of the invention

The oligomerization domains were selected for having a fairly
small molecular weight and for being compatible with trans-
port of the fusion protein through the membrane. They are
based on two different types of amphiphilic helices.

Amphiphilic helices are known to predominantly, but not
exclusively, associate in two different molecular structures:
Four helix bundles and coiled coils. The design and formation
of helix bundles has been studied previously (Eisenberg et
al., 1986, Proteins 1, 16-22; Ho and deGrado, 1987, J. Am.
Chem. Soc. 109, 6751-6758; Regan and deGrado, 1988, Science
241, 976-978 ; Hill et al., 1990, Science 294, 543-546).
This molecule association is also known from natural proteins
(Richardson, 1981, Adv. Prot. Chem. 34, 167).

The four helix bundle may be formed from either four separate
molecules (each contributing one helix), two molecules con-
taining two helices each (connected as helix-turn-helix) or
one molecule containing a helix-turn-helix-turn-helix-turn-
helix motif. For dimerization or multimerization, only the
first two are suitable.

Three variations of this latter theme were tested. In the
first, one helix of the sequence given in Eisenberg et al.
(1986) (Proteins 1, 16-22) was used. In the second, this
sequence was extended by a small hydrophilic peptide ending
in a cysteine. Once the helices are associated, the hydrophi-
lic peptides are held in sufficiently close contact that they

can collide and a disulfide bond can form under oxidizing conditions, as in the periplasm of E. coli. In the third variation, two helices are used in tandem, separated by a short turn encoding peptide.

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In the second design, peptides are used which can form so-called coiled-coil structures. Such peptides occur in transcription factors such as e. g. GCN4 from yeast and have been called leucine zippers (Landschulz et al., 1988, Science

10   240, 1759-1764). The crystal structure of this has been solved recently (O'Shea et al., 1991, Science 254, 539-544) and showed a parallel arrangement of the helices.

A covalent attachment of the helices is possible by a small

15   peptide extension, again containing a cystein. Since the helices are now parallel, the peptide extension can be much shorter, since the distance is much smaller.

The various dimerization devices (intercalating helices) were

20   however not fused to the antibody domain directly. It is advantageous to introduce a flexible peptide between the end of the scFv fragment and the beginning of the helix. As an example, the upper hinge region of mouse IgG3 has been used. However, a variety of hinges can be used. It is not required

25   for dimerization per se, but provides a spacing of the two scFv domains similar to the antigen binding sites of a whole antibody. This way, the two binding sites span a greater distance in space and therefore can reach neighboring antigens on a solid surface.

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The naturally occuring hinges of antibodies are preferred
embodiments of hinges in bivalent miniantibodies. In the case
of bifunctional miniantibodies, the hinges may be shorter,
since frequently molecules from different surfaces are to be

5   crosslinked as close as possible, and flexibilty of the dimer
is not necessary. The choice of the hinge is governed by the
desired residue sequence,1 ength (Argos, 1990, J. Mol. Biol.
211, 943-958), compatibility with folding and stability of
the amphiphilic helices (Richardson & Richardson, 1988,

10   Science 240, 1648-1652), secretion and resistance against
proteases .

The present invention deals with peptides as dimerization
devices, which should be as small as possible. One preferred

15   embodiment is the use of peptides which can form amphipathic
helices. Such helices shield the hydrophobic surface by
dimerization or even multimerization. Helices of this type
are characterized by their having hydrophobic patches on one
face of the helix, and containing a sufficient number of

20   helix-forming residues. Rules for such peptides are discussed
in Eisenberg et al. 1986, O'Shea et al.,.1991 (Science 254,
539-544),1992 (Cell 68, 699-708).

Natural peptides of this type are found as the so-called

25   leucine zippers, characterized by a periodic occurence of
leucine (every seventh residue) and other hydrophilic resi-
dues (e. g. valine) also every seventh residue. As these
priniples are now understood (O'Shea et al. 1991, 1992,
literature cited), the sequence can be varied to incorporate

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residues which make the association of homodimers unfavor-
able, but favors the association of heterodimers. Such
sequence alteration can e. g. involve the incorporation of
charge bridges, such that in the homodimers, like charges

5   repel each other and in the heterodimer, opposite charges
attract each other (see below).

The present invention can also be extended to bifunctional
miniantibodies. In this case, dimerization devices (inter-

10   calation peptides) have to be used which will only allow the
formation of heterodimers, but not homodimers. A preferred
embodiment of this part of the invention are two different
coiled-coil helices, such as in naturally occuring leucine
zippers, e. g. from the transcription factor proteins jun and

15   fos (O'Shea et al.,1989, Science 245, 646-648).

In a further embodiment of the invention, the constant scFv-
hinge-helix can be extended at the C-terminus to result in a
fusion protein. For example, a fusion to an enzyme may be

20   made to use such bivalent constructs in diagnostics. Such
enzymes are e. g. alkaline phosphatase, luciferase or horse
radish peroxidase. The advantage of such a antibody-enzyme
fusion protein would be that the bivalence of the antibody
would lead to an enhanced binding to the surface-bound anti-

25   gen. The advantage over a fusion protein prepared by conven-
tional technology (i. e. chemical coupling of the antibody to
the enzyme of choice) would be a greater batch-to-batch
consistency; homogeneity of the product and the much simpler
method of preparation, namely from E. coli in a single step.

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In the same fashion, the miniantibodies may be extended at
the C-terminus to incorporate a toxin. Such immunotoxins
would be bivalent or even bispecific and thus combine the
advantages of such antibody fragments linked above with the
5   advantages in tumor therapy known for immunotoxins. Simi-
larly, a metal binding peptide or protein could be linked
genetically to be used in radioimmunotherapy or in tumor
imaging. The same advantages for any genetically encoded
hybrid protein hold true as given above for the antibody-en-
10   zyme fusions.

In another embodiment of the invention, a construct of the
type scFv-hinge-helix may be made to dimerize with another
protein fused to a dimerization domain, in complete analogy
15   as described above for the formation of bispecific minianti-
bodies. In this fashion, the scFv fragment would e. g. be
fitted with the helix of the fos protein. Such foreign pro-
tein, which could be made to form heterodimers with the scFv
fragment, include enzymes useful in diagnosis, toxins, metal-
20   binding peptides or proteins useful in radioimmunotherapy or
radio-imaging.

Using the principles of this invention, the dimerization
domains presented here can also serve for purification purpo-
25   ses. A recombinant protein of any kind can be fused to a
dimerization domain, e.g. to hinge-fos-zipper. After coex-
pression with a scFv-hinge-jun, the heterodimer can be puri-
fied in one step with an affinity column for the scFv-speci-
ficity. In an alternative approach, the 'opposite' zipper,
30   linked to a column support, 'catches' the protein-hinge-zip-
per when passing through the column as a crude cell extract.

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The elution of the pure fusion protein from the column is
possible using the unfolding temperature of the zipper. A
subsequent separation from the dimerization domain is achie-
vable by introduction of a proteolytic site, e.g. for blood
5   clotting factor Xa, into the hinge (Nagai & Thogerson, 1987,
Meth. Enyzmol. 152,,461-481).

A particular advantage of the miniantibodies described in
this invention is the ability to assemble functionally in
10   Escherichia coli. In the case of homobivalent constructs, a
dimerization principle is used which allows the formation of
homodimers. Examples described above include the coiled-coil
helix (leucine zipper) of the yeast protein GCN4 or the
helices from an antiparallel 4-helix bundle. In this case,
15   the scFv fragment is expressed in the presence of a bacterial
signal sequence and carries at the end of the gene of the
scFv fragment the codons for a hinge and the dimerization
helix or helix-turn-helix. The helices are compatible with
secretion to the periplasmic space in E. coli, where protein
20   folding, disulfide formation and assembly occurs. Under these
conditions, the homodimeric proteins form by themselves and
can directly be isolated in the dimeric form.

If heterobivalent constructs are desired, two different scFv
25   fragments or one scFv fragment associating with a different
protein need to associate. In the preferred embodiment of
this invention, both proteins to be assembled are expressed
in the same cell, preferably on the same plasmid, preferably
as a dicistronic operon. The design of artificial dicistronic

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operons is explained e.g. in Skerra et al. (1991, Protein Eng. 4, 971). Since the assembly must take place in the periplasm, because the scFv fragment can only fold in the oxidizing milieu, both proteins must be transported and both

5   must be fitted with a signal sequence. The dimerization peptides must be chosen such that they promote the associa- tion of two different proteins, but prevent the association of the respective homodimers. Examples of such proteins are the leucine zipper peptides of the proteins fos and jun (see

10   above).

When not expressed in the same cell, the different scFv- hinge-zipper constructs have to be mixed together as a crude cell extract or purified protein and treated with raised

15   temperature. In absence of the 'opposite' zipper, e.g. a scFv-hinge-jun-zipper construct is able to form homodimers. After short heating to the melting temperature of around 40-C, the zippers of the unwanted homodimer unfold and form a much more stable heterodimer (O'Shea et al., 1992, Cell 68,

20   699-708). Without raising the temperature, formation of heterodimers in vitro is not possible, as tested in experi- ments.

Short Description of the Figures and the Sequence Listing

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Fig. 1   scFv-Expression vector pLISC-SE containg the scFv- fragment.

Fig. 2   Dicistronic scFv-hinge-zipper expression vector

30   pACKxFyJ.

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Fig. 3   Functional ELISA;

The concentrations of the affinity purified proteins, measured by OD_{280} (vertical axis), refer to the molar number of binding sites per well (horizontal axis). The ELISA plates were coated with phosphocholine-BSA, and the purified phosphocholine-specific miniantibody-proteins were bound and detected by an anti-McPC603 antiserum.

(a) Comparison of various miniantibodies.

(b) Comparison of miniantibody scHLXc with ScFV and whole IgA.

Fig. 4   Functional Anti-lysozyme ELISA;
PC-affinity purified samples of coexpressed anti-PC-anti-lysozyme bispecific miniantibody. + and - on the horizontal axis means: plus inhibitor (+) and without inhibitor (-).

The attached sequence listing refers to sequence identity numbers (S.I.N.):

S.I.N. 1:   Whole nucleotid- and amino acid sequence of the pLISC-SE vector.

S.I.N. 2:   Gene cassette of intercalating GCN4-leucine zipper (nucleotid- and amino acid sequence).

S.I.N. 3:   Gene cassette encoding intercalating antiparallel helix-turn-helix (nucleotid- and amino acid sequence).

S.I.N. 4:   Gene cassette encoding intercalating jun-zipper
             and IgG3-hinge region.

S.I.N. 5:   Gene cassette encoding intercalating fos-zipper
5   and IgG3-hinge region.

S.I.N. 6:   Gene cassette encoding intercalating jun-zipper
             and designed linker.

10   S.I.N. 7:   Gene cassette encoding intercalating fos-zipper
                  and designed linker.

Example 1:   Construction of vectors for secreted single-chain
             fragments, containing a restriction site for
15   introducing genes for intercalating peptides.

Recombinant DNA-techniques were based on Sambrook et al.
(1989, Molecular Cloning: A laboratory manual. Second edi-
tion. Cold Spring Harbor Laboratory, New York). Functional
20   expression of the single-chain Fv fragments and the minian-
tibodies in E. coli JM83 was carried out with vectors
similar to pASK-lisc (Skerra et al., 1991, Protein Eng. 4,
971). Site directed mutagenesis was directly performed in
these vectors according to Kunkel et al. (1987, Meth. Enzy-
25   mol. 154, 367-382) and Geisselsoder et al. (1987, Biotechni-
ques 5, 786-791) using the helper phage M13K07 (Vieira &
Messing, 1987, Meth. Enzymol. 153, 3-11). SDS-PAGE was car-
ried out as described by Fling and Gregerson (1986, Anal.
Biochem. 155, 83-88). Concentrations of affinity-purified
30   proteins were measured by OD_{280} using calculated extinction

coefficients (Gill & von Hippel, 1989, Anal. Biochem. 182, 319-326).A vector such as pASK40 (Skerra et al., 1991, Protein Eng. 4, 971) is used, which contains an origin of replication, a regulatable promotor, a bacterial signal sequence followed by a multiple cloning site, a transcription terminator and an origin for single stranded phages.The gene for the single-chain Fv fragment is designed as follows: The nucleotide sequence of a V_H domain is directly followed by a linker sequence encoding preferably about 15 residues, preferably of the sequence $(Gly_4Ser)_3$, followed directly by the sequence of the V_L domain. Alternatively, the sequence of the V_L domain may be directly followed by the sequence of the linker, followed by the sequence of the V_H domain.

If the antibody is of known sequence, the complete gene of the scFv fragment may be assembled from synthetic oligonucleotides. A detailed experimental procedure for such a gene synthesis of an antibody gene is e.g. given in Plückthun et al. (1987, Cold Spring Harbor Symp. Quant. Biol. 52, 105-112).

If the genes of the V_H and V_L domains are present in other vectors, the gene for the scFv fragment may be assembled from restriction fragments. For example, a restriction fragment encoding most of the V_H domain may be excised from another plasmid, and a fragment encoding most of the V_L domain may be excised from a plasmid. The remaining pieces of V_L and V_H and

the linker for the scFv fragment can be provided by cassettes
of synthetic oligonucleotides, which need to be ligated by
standard methodology (Sambrook et al., 1989, literature
cited). The mixture of fragments is ligated into the vector

5   pASK40 or a similar plasmid containing a pair of suitable
restriction sites.

If the genes of the antibody have not been cloned before,
they may be directly obtained from the hybridoma cell pro-

10   ducing the antibody by the polymerase chain reaction (PCR;
PCR methodology is described in McPherson et al., 1991, PCR-A
Practical Approach Oxford University Press, New York). Pri-
mers suitable for amplification of V_H and V_L domains have been
given by Orlandi et al., 1989, Proc. Natl. Acad. Sci. USA 86,

15   3833-3837; Huse et al., 1989, Science 246, 1275-1281; Larrick
et al., 1989, Bio-technology 7, 934-938. The methodology of
obtaining mRNA from hybridoma is described in these referen-
ces as well. The separate V_H and V_L genes may be cloned into
separate vectors, and the scFv gene assembled according to

20   the principles explained above.

If the ligated fragments do not result in a correct reading
frame of the scFv fragment, a precise fusion with the signal
sequence codons resident on the plasmid may be generated by

25   site directed mutagenesis. The design of the oligonucleotides
and the execution is possible for anyone skilled in the art.

30

21

The scFv expression plasmid so obtained contains the codons
for a bacterial signal sequence, directly followed by the
first variable domain (V_H or V_L), a linker and the second
variable domain (V_L or V_H) under the control of a regulatable
5   promotor.

At the 3' end of this genes, corresponding to the C-terminus
of the scFv protein, a unique restriction site is introduced
into the expression plasmid to allow insertion of cassettes
10 coding for the intercalating peptides. The restriction site
is introduced by site directed mutagenesis using the method
of Kunkel (1987, Meth. Enzymol. 154, 367-382).

An example of the complete sequence of a suitable single-
15 chain Fv expression plasmid pLISC-SE for receiving an inter-
calation peptide is shown in Fig. 1 and Sequence Identity No.
(S.I.N.) 1 (see Squence Listing).

20 **Example 2:**   Design and construction of a gene cassette enco-
                    ding intercalating peptides of a leucine zipper.

The gene cassette, fitted with restriction sites to be compa-
tible with the restriction site at the 3' end of the scFv
25 fragment gene, must encode the sequence of a hinge (con-
nection the scFv fragment to the intercalating peptide) and
the intercalation peptide itself. The hinge region, may
however also be obmitted.
As an example the sequence of the upper hinge region of mouse
30 IgG3 (Dangl et al., 1988, EMBO J. 7, 1989-1994), followed by
the sequence of the leucine zipper sequence of the yeast
protein GCN4 (Oas et al., 1990, Biochemistry T29, 2891-2894),

2 2

is back-translated into frequently used E. coli codons
(S.I.N.: 2). Oligonucleotides are synthesized, and ligated
into the vector pLISC-SE, previously digested with EcoRI and
Hind III.

5

Example 3:   Design and construction of a gene cassette enco-
             ding intercalating peptides of a four-helix
             bundle.

10   Analogous to Example 2, the sequence of the upper hinge
     region of mouse IgG3, followed by the sequence of the helix-
     turn-helix of a four helix bundle (Eisenberg et al., 1986,
     literature cited) is backtranslated into frequently used E.
     coli codons (S.I.N.: 3). Oligonucleotides are synthesized,
15   and ligated into the vector pLISC-SE, previously digested
     with Eco RI and Hind III.

Example 4:   Design and construction two gene cassettes enco-
             ding intercalating peptides of a leucine zipper
20   and their co-expression.

     Analogous to Example 2, the sequence of the upper hinge
     region of mouse IgG3 followed by the sequence of the zipper
     sequence of the jun protein (O'Shea et al., 1992, literature
25   cited), is backtranslated into frequently used E. coli codons
     (S.I.N.: 4). Oligonucleotides are synthesized, and ligated
     into the vector pLISC-SE, previously digested with EcoRI and
     Hind III.

30

23

In a parallel reaction, the sequence of the upper hinge
region of mouse IgG3, followed by the sequence of the zipper
sequence of the fos protein (O'Shea et al., 1992, Cell 68,
699-708), is backtranslated into frequently used E. coli
5   codons (S.I.N.: 5). Oligonucleotides are synthesized, and
ligated into the vector pLISC-SE, previously digested with
Eco RI and Hind III.The two vectors thus each code for a
different antibody scFv fragment, followed by a hinge peptide
and a different leucine zipper peptide. To co-express the two
10   scFv fragments, the whole scFv-hinge-zipper gene of the
fos-containing product is excised from the vector as a Xba
I-Hind III fragment and ligated into the vector, pLISC-SE-
scFv-jun, containing already the scFv gene of the other
antibody.
15   The newly obtained vector then expresses the $scFv_1$-$linker_1$-
fos-zipper and the $scFv_2$-$linker_2$-jun-zipper from a single
promoter as a dicistronic operon.

An improved sequence for the hinge region in the context of
20   fos and jun zippers is given in S.I.N.: 6 and 7. This hinge
is shorter and therefore not as susceptible to proteolysis.
In cases, where the distance between the two binding sites is
of less importance, such shortened hinges may be advanta-
geous. In this case, the "tail" of the scFv fragment has been
25   shortened and the EcoRI site, which receive the genes for the
intercalating peptides has been moved four residues upstream.

30

2 4

Example 5:   Purification of bivalent miniantibody from E.
             coli.

E. coli JM83, harboring a plasmid constructed as in examples
5   II and III, are grown to an O.D. 550 of 0.5 and induced with
     IPTG at a final concentration of 1 mM. The cells are centri-
     fuged, resuspended in BBS buffer (200 mM Na-borate, 160 mM
     NaCl, pH 8.0) and the suspension is passed through a French
     press. In these examples, a phosphorylcholine binding minian-
10   tibody is used. The miniantibody is purified by passage over
     a phosphorylcholine affinity chromatography as described
     (Chesebro and Metzger, 1972, Biochemistry 11, 766-771).

     Example 6:   Purification of a bispecific miniantibody from E.
15   coli

E. coli JM83, harboring a plasmid constructed as in examples
II and III and containing a dicistronic structural gene for
two different scFv (Fig. 2), are grown to an O.D. 550 of 0.5
20   and induced with IPTG at a final concentration of 1mM. The
     cells are centrifuged, resuspended in BBS buffer (200 mM
     Na-borate, 160 mM NaCl, pH 8.0) and the suspension is passed
     through a French press.
     In this example, a bispecific miniantibody is used containing
25   both a specificity for phosphorylcholine as well as benzoyl-
     ampicillin. The miniantibody is purified by passage over a
     phosphorylcholine affinity chromatography as described (Che-
     sebro and Metzger, 1972, literature cited).

30

2 5

Example 7:   Surface binding of bivalent miniantibodies

The ELISA-plates (Nunc, Macrosorp) were coated with 400 ~g/ml
phosphocholine-BSA in PBS buffer (20 mM phosphate, pH 7.2,
5   115 mM NaCl). The hapten reagent was prepared from nitro-
phenyl phosphocholine (Sigma), which was reduced and diazoti-
zed essentially as described (Chesebro & Metzger, 1972,
literature cited), and reacted by azo-coupling to BSA (Sigma)
in borate-saline buffer (52.5 mM sodium borate, pH 9, 120 mM
10   NaCl) at 4~C for 48 hours with subsequent dialysis against
PBS. After blocking the non-coated plate surface with 5% skim
milk (Nestle) in PBS buffer for at least 2 hours, the peri-
plasmic extract or the purified protein was incubated in BBS
buffer on the plate for 90 min at room temperature. After
15   thorough washing (3 times), remaining functional antibody
fragments were detected according standard procedures (Harlow
& Lane, 1988, "Antibodies, A Laboratory Manual", Cold Spring
Harbor Laboratory, 555-592) with rabbit anti-McPC603 serum
and anti-rabbit immunoglobulin linked to peroxidase (Sigma)
20   according to Gallati (1979, Clin. Chem. Clin. Biochem. 17,
1-4).

An enormous gain in binding, and thus sensitivity, is obser-
ved for all miniantibody constructs, compared to the monome-
25   ric scFv fragment. This is consistent with the simultaneous
binding of two or even more binding sites to the same sur-
face. _These avidity of the fusion protein scHLXc was compa-
rable to the natural antibody McPC603, which could be
detected with antigen-coated ELISA, while the monomeric scFv
30   fragment could only be detected with hundred-fold higher
concentrations (Fig. 3 a, b). All binding is nearly totally

2 6

inhibitible with soluble hapten, except of the monomeric scFv
fragment. The thermodynamic affinity of the natural antibody
to soluble phosphocholine is about 1.6 $ 10⁵ M⁻¹ and thus
relatively weak (Metzger et al., 1971, Proceedings of the I
5   st Congress of Imunology. Academic Press, New York, pp.
253-267), and this is apparently not sufficient for a monome-
ric fragment-hapten complex to survive the repeated washing
steps of a functional ELISA (Kemeny & Challacombe, 1988,
"ELISA and other solid phase immunoassays", Wiley & Sons, New
10   York).

Example 8:   Surface binding of bifunctional miniantibodies

Coexpressed bifunctional miniantibodies recognizing phos-
15   phorylcholine with one arm and lysozyme with the other arm
were purified by phosphocholine (PC) affinity chromatography
and tested for lysozyme specificity. An ELISA-plate was
coated with lysozyme, the ELISA was carried out as described
in Example VII.Three different preparations show binding to
20   the antigen-surface, which is completely inhibitible with
soluble lysozyme (Fig. 4).

25

30

SEQUENCE LISTING:

SEQ ID NO: 1

```
ACCCGACACC ATCGAATGGC GCAAAACCTT TCGCGGTATG GCATGATAGC    50
GCCCGGAAGA GAGTCAATTC AGGGTGGTGA ATGTGAAACC AGTAACGTTA    100
TACGATGTCG CAGAGTATGC CGGTGTCTCT TATCAGACCG TTTCCCGCGT    150
GGTGAACCAG GCCAGCCACG TTTCTGCGAA AACGCGGGAA AAAGTGGAAG    200
CGGCGATGGC GGAGCTGAAT TACATTCCCA ACCGCGTGGC ACAACAACTG    250
GCGGGCAAAC AGTCGTTGCT GATTGGCGTT GCCACCTCCA GTCTGGCCCT    300
GCACGCGCCG TCGCAAATTG TCGCGGCGAT TAAATCTCGC GCCGATCAAC    350
TGGGTGCCAG CTGTGTGGTG TCGATGGTAG AACGAAGCGG CGTCGAAGCC    400
TGTAAAGCGG CGGTGCACAA TCTTCTCGCG CAACGCGTCA GTGGGCTGAT    450
CATTAACTAT CCGCTGGATG ACCAGGATGC CATTGCTGTG GAAGCTGCCT    500
GCACTAATGT TCCGGCGTTA TTTCTTGATG TCTCTGACCA GACACCCATC    550
AACAGTATTA TTTTCTCCCA TGAAGACGGT ACGCGACTGG GCGTGGAGCA    600
TCTGGTCGCA TTGGGTCACC AGCAAATCGC GCTGTTAGCG GGCCCATTAA    650
GTTCTGTCTC GGCGCGTCTG CGTCTGGCTG GCTGGCATAA ATATCTCACT    700
CGCAATCAAA TTCAGCCGAT AGCGGAACGG GAAGGCGACT GGAGTGCCAT    750
GTCCGGTTTT CAACAAACCA TGCAAATGCT GAATGAGGGC ATCGTTCCCA    800
CZGCGATGCT GGTTGCCAAC GATCAGATGG CGCTGGGCGC AATGCGCGCC    850
ATTACCGAGT CCGGGCTGCG CGTTGGTGCG GATGTCTCGG TAGTGGGATA    900
CGCAGATACC GAAGACAGCT CATGTTATAT CCCGCCGTTA ACCACCATCA    950
AACAGGATTT TCGCCTGCTG GGGCAAACCA GCGTGGACCG CTTGCTGCAA    1000
CTCTCTCAGG GCCAGGCGGT GAAGGGCAAT CAGCTGTTGC CCGTCTCACT    1050
GGTGAAAAGA AAAACCACCC TGGCGCCCAA TACGCAAACC GCCTCTCCCC    1100
GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG    1150
GAAAGCGGGC AGTGAGCGCA ACGCAATTAA TGTGAGTTAG CTCACTCATT    1200
AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT GTTGTGTGGA    1250
ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACCATGATTA    1300
CGAATTTCTA GATAACGAGG GCAAAAA---ATG AAA AAG ACA GCT ATC    1345
                                   Met Lys Lys Thr Ala Ile
                                    1                  5
GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG    1387
Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln
            10                  15                  20
GCC GAA GTT AAA CTG GTA GAG TCT GGT GGT GGT CTG GTA CAG    1429
Ala Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln
                25                  30
CCG GGT GGA TCC CTG CGT CTG TCT TGC GCT ACC TCA GGT TTC    1471
Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Phe
 35                  40                  45
ACC TTC TCT GAC TTC TAC ATG GAG TGG GTA CGT CAG CCC CCG    1513
Thr Phe Ser Asp Phe Tyr Met Glu Trp Val Arg Gln Pro Pro
     50                  55                  60
GGT AAA CGT CTC GAG TGG ATC GCA GCT AGC CGT AAC AAA GGT    1555
Gly Lys Arg Leu Glu Trp Ile Ala Ala Ser Arg Asn Lys Gly
         65                  70                  75
AAC AAG TAT ACC ACC GAA TAC AGC GCT TCT GTT AAA GGT CGT    1597
Asn Lys Tyr Thr Thr Glu Tyr Ser Ala Ser Val Lys Gly Arg
             80                  85                  90
```

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TTC ATC GTT TCT CGT GAC ACT AGT CAA TCG ATC CTG TAC CTG       1639
Phe Ile Val Ser Arg Asp Thr Ser Gln Ser Ile Leu Tyr Leu
                95                      100
CAG ATG AAT GCA TTG CGT GCT GAA GAC ACC GCT ATC TAC TAC       1681
Gln Met Asn Ala Leu Arg Ala Glu Asp Thr Ala Ile Tyr Tyr
105                 110                 115
TGC GCG CGT AAC TAC TAT GGC AGC ACT TGG TAC TTC GAC GTT       1723
Cys Ala Arg Asn Tyr Tyr Gly Ser Thr Trp Tyr Phe Asp Val
        120                 125                 130
TGG GGT GCA GGT ACC ACC GTT ACC GTT TCT TCT GGT GGT GGT       1765
Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
            135                 140                 145
GGT TCT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GAT ATC       1807
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile
            150                 155                 160
GTT ATG ACC CAG TCT CCG AGC TCT CTG TCT GTA TCT GCA GGT       1849
Val Met Thr Gln Ser Pro Ser Ser Leu Ser Val Ser Ala Gly
                165                 170
GAA CGT GTT ACC ATG TCT TGC AAA TCT TCT CAG TCT CTG CTG       1891
Glu Arg Val Thr Met Ser Cys Lys Ser Ser Gln Ser Leu Leu
175                 180                 185
AAC TCT GGT AAC CAG AAA AAC TTC CTG GCG TGG TAT CAG CAA       1933
Asn Ser Gly Asn Gln Lys Asn Phe Leu Ala Trp Tyr Gln Gln
        190                 195                 200
AAG CCT GGC CAA CCG CCG AAA CTG CTG ATC TAC GGT GCG TCG       1975
Lsy Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser
        205                 210                 215
ACC CGT GAA TCT GGT GTT CCG GAC CGT TTT ACC GGT AGC GGT       2017
Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
            220                 225                 230
AGC GGT ACC GAC TTC ACT CTG ACC ATC TCT TCT GTA CAG GCT       2059
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala
            235                 240
GAA GAT CTG GCT GTT TAC TAC TGT CAA AAC GAC CAC TCT TAC       2101
Glu Asp Leu Ala Val Tyr Tyr Cys Gln Asn Asp His Ser Tyr
245                 250                 255
CCG CTG ACC TTT GGC GCC GGC ACC AAA CTG GAA CTG AAG CGC       2143
Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
        260             · 265                 270
GCT AAC GGT GAA TTC-TGATAAGCTTGA CCTGTGAAGT GAAAAATGGC       2190
Ala Asn Gly Glu Phe *
        275
GCACATTGTG CGACATTTTT TTTGTCTGCC GTTTACCGCT ACTGCGTCAC       2240
GGATCCCCAC GCGCCCTGTA GCGGCGCATT AAGCGCGGCG GGTGTGGTGG       2290
TTACGCGCAG CGTGACCGCT ACACTTGCCA GCGCCCTAGC GCCCGCTCCT       2340
TTCGCTTTCT TCCCTTCCTT TCTCGCCACG TTCGCCGGCT TTCCCCGTCA       2390
AGCTCTAAAT CGGGGCATCC CTTTAGGGTT CCGATTTAGT GCTTTACGGC       2440
ACCTCGACCC CAAAAAACTT GATTAGGGTG ATGGTTCACG TAGTGGGCCA       2490
TCGCCCTGAT AGACGGTTTT TCGCCCTTTG ACGTTGGAGT CCACGTTCTT       2540
TAATAGTGGA CTCTTGTTCC AAACTGGAAC AACACTCAAC CCTATCTCGG       2590
TCTATTCTTT TGATTTATAA GGGATTTTGC CGATTTCGGC CTATTGGTTA       2640
```

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```
AAAAATGAGC  TGATTTAACA  AAAATTTAAC  GCGAATTTTA  ACAAAATATT    2690
AACGTTTACA  ATTTCAGGTG  GCACTTTTCG  GGGAAATGTG  CGCGGAACCC    2740
CTATTTGTTT  ATTTTTCTAA  ATACATTCAA  ATATGTATCC  GCTCATGAGA    2790
CAATAACCCT  GATAAATGCT  TCAATAATAT  TGAAAAAGGA  AGAGTATGAG    2840
TATTCAACAT  TTCCGTGTCG  CCCTTATTCC  CTTTTTTGCG  GCATTTTGCC    2890
TTCCTGTTTT  TGCTCACCCA  GAAACGCTGG  TGAAAGTAAA  AGATGCTGAA    2940
GATCAGTTGG  GTGCACGAGT  GGGTTACATC  GAACTGGATC  TCAACAGCGG    2990
TAAGATCCTT  GAGAGTTTTC  GCCCCGAAGA  ACGTTTTCCA  ATGATGAGCA    3040
CTTTTAAAGT  TCTGCTATGT  GGCGCGGTAT  TATCCCGTAT  TGACGCCGGG    3090
CAAGAGCAAC  TCGGTCGCCG  CATACACTAT  TCTCAGAATG  ACTTGGTTGA    3140
GTACTCACCA  GTCACAGAAA  AGCATCTTAC  GGATGGCATG  ACAGTAAGAG    3190
AATTATGCAG  TGCTGCCATA  ACCATGAGTG  ATAACACTGC  GGCCAACTTA    3240
CTTCTGACAA  CGATCGGAGG  ACCGAAGGAG  CTAACCGCTT  TTTTGCACAA    3290
CATGGGGGAT  CATGTAACTC  GCCTTGATCG  TTGGGAACCG  GAGCTGAATG    3340
AAGCCATACC  AAACGACGAG  CGTGACACCA  CGATGCCTGT  AGCAATGGCA    3390
ACAACGTTGC  GCAAACTATT  AACTGGCGAA  CTACTTACTC  TAGCTTCCCG    3440
GCAACAATTA  ATAGACTGGA  TGGAGGCGGA  TAAAGTTGCA  GGACCACTTC    3490
TGCGCTCGGC  CCTTCCGGCT  GGCTGGTTTA  TTGCTGATAA  ATCTGGAGCC    3540
GGTGAGCGTG  GGTCTCGCGG  TATCATTGCA  GCACTGGGGC  CAGATGGTAA    3590
GCCCTCCCGT  ATCGTAGTTA  TCTACACGAC  GGGGAGTCAG  GCAACTATGG    3640
ATGAACGAAA  TAGACAGATC  GCTGAGATAG  GTGCCTCACT  GATTAAGCAT    3690
TGGTAACTGT  CAGACCAAGT  TTACTCATAT  ATACTTTAGA  TTGATTTAAA    3740
ACTTCATTTT  TAATTTAAAA  GGATCTAGGT  GAAGATCCTT  TTTGATAATC    3790
TCATGACCAA  AATCCCTTAA  CGTGAGTTTT  CGTTCCACTG  AGCGTCAGAC    3840
CCCGTAGAAA  AGATCAAAGG  ATCTTCTTGA  GATCCTTTTT  TTCTGCGCGT    3890
AATCTGCTGC  TTGCAAACAA  AAAAACCACC  GCTACCAGCG  GTGGTTTGTT    3940
TGCCGGATCA  AGAGCTACCA  ACTCTTTTTC  CGAAGGTAAC  TGGCTTCAGC    3990
AGAGCGCAGA  TACCAAATAC  TGTCCTTCTA  GTGTAGCCGT  AGTTAGGCCA    4040
CCACTTCAAG  AACTCTGTAG  CACCGCCTAC  ATACCTCGCT  CTGCTAATCC    4090
TGTTACCAGT  GGCTGCTGCC  AGTGGCGATA  AGTCGTGTCT  TACCGGGTTG    4140
GACTCAAGAC  GATAGTTACC  GGATAAGGCG  CAGCGGTCGG  GCTGAACGGG    4190
GGGTTCGTGC  ACACAGCCCA  GCTTGGAGCG  AACGACCTAC  ACCGAACTGA    4240
GATACCTACA  GCGTGAGCTA  TGAGAAAGCG  CCACGCTTCC  CGAAGGGAGA    4290
AAGGCGGACA  GGTATCCGGT  AAGCGGCAGG  GTCGGAACAG  GAGAGCGCAC    4340
GAGGGAGCTT  CCAGGGGGAA  ACGCCTGGTA  TCTTTATAGT  CCTGTCGGGT    4390
TTCGCCACCT  CTGACTTGAG  CGTCGATTTT  TGTGATGCTC  GTCAGGGGGG    4440
CGGAGCCTAT  GGAAAAACGC  CAGCAACGCG  GCCTTTTTAC  GGTTCCTGGC    4490
CTTTTGCTGG  CCTTTTGCTC  ACATG                                 4515
```

30

SEQ ID NO: 2

```
GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CGC ATG 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Met
1   ↑             5                  10                    15
        L_____ IgG3-hinge _____J L_____
AAA CAG CTG GAA GAT AAA GTT GAA GAG CTT CTT TCG AAA AAC TAC 90
Lys Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr
             20                  25                    30
_____ GCN4-zipper _____
CAC CTC GAA AAT GAA GTT GCG CGC CTC AAA AAA CTT GTT GGT GAA 135
His Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu
             35                  40                    45
_____
CGC TGATAAGCTT GAC                                          151
Arg         ↑
___stop
```

SEQ ID NO: 3

```
GGT GAA TTC CCC AAA CCT AGC ACC CCC CCT GGC AGC AGT GGT GAA 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Gly Glu
1   ↑             5                  10                    15
        L_____ IgG3-hinge _____J L_____
CTG GAA GAG CTG CTT AAG CAT CTT AAA GAA CTT CTG AAG GGC CCC 90
Leu Glu Glu Leu Leu Lys His Leu Lys Glu Leu Leu Lys Gly Pro
             20                  25                    30
_____ bundle-helix A _____J L__
CGC AAA GGC GAA CTC GAG GAA CTG CTG AAA CAT CTG AAG GAG CTG 135
Arg Lys Gly Glu Leu Glu Glu Leu Leu Lys His Leu Lys Glu Leu
             35                  40                    45
_turn__J L_____ bundle-helix B _____
CTT AAA GGT GAA TTC TGATAAGCTT GACCTGTGAA GTGAAAAAAT G      191
Leu Lys Gly Glu Phe
             ↑    50        ↑
_____J stop
```

SEQ ID NO: 4

```
GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CGT ATC 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Ile
1    ↑             5                      10                  15
        L------IgG3-hinge--------------------------------J L------
GCT CGT CTC GAG GAA AAA GTT AAA ACC CTG AAA GCT CAG AAC TCC 90
Ala Arg Leu Glu Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser
                    20                  25                  30
------------------------- jun-zipper ---------------------------
GAA CTG GCT TCC ACC GCT AAC ATG CTG CGT GAA CAG GTT GCT CAG 135
Glu Leu Ala Ser Thr Ala Asn Met Leu Arg Glu Gln Val Ala Gln
                    35                  40                  45
------------------------- jun-zipper ---------------------------
CTG AAA CAG AAA GTT ATG AAC TAC TGATAAGCTT GACCTGTGAA G       180
Leu Lys Gln Lys Val Met Asn Tyr                  ↑
                    50                          stop
----------------------------------------J
```

SEQ ID NO: 5

```
GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CTG ACC 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Leu Thr
1    ↑             5                      10                  15
        L----------------- IgG3-hinge -----------------J L------
GAC ACC CTG CAG GCT GAA ACC GAC CAG CTG GAA GAC AAA AAA TCC 90
Asp Thr Leu Gln Ala Glu Thr Asp Gln Leu Glu Asp Lys Lys Ser
                    20                  25                  30
------------------------- fos-zipper ---------------------------
GCT CTG CAG ACC GAA ATC GCT AAC CTG CTG AAA GAA AAA GAA AAA 135
Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys
                    35                  40                  45
------------------------- fos-zipper ---------------------------
CTG GAA TTT ATC CTG GCT GCT TAC TGATAAGCTT GACCTGTGAA G       180
Leu Glu Phe Ile Leu Ala Ala Tyr                  ↑
                    50                          stop
----------------------------------J
```

SEQ ID NO: 6

```
GGT GAA TTC CCG TCT GGT AAC GAA GCT CGT ATC GCT CGT CTC GAG  45
Gly Glu Phe Pro Ser Gly Asn Glu Ala Arg Ile Ala Arg Leu Glu
1    ↑           5                   10                   15
     L----- linker ------------┘ L------------------------------
GAA AAA GTT AAA ACC CTG AAA GCT CAG AAC TCC GAA CTG GCT TCC  90
Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser Glu Leu Ala Ser
                    20                  25                  30
--------------------------- jun-zipper ---------------------------
ACC GCT AAC ATG CTG CGT GAA CAG GTT GCT CAG CTG AAA CAG AAA  135
Thr Ala Asn Met Leu Arg Glu Gln Val Ala Gln Leu Lys Gln Lys
                    35                  40                  45
--------------------------- jun-zipper --------------------------
GTT ATG AAC TAC TGATAAGCTT GACCTGTGAA GTGAAAAATG GCG          180
Val Met Asn Tyr          ↑
---------------┘ stop
```

SEQ ID NO: 7

```
GGT GAA TTC GGT CCG TCT GGT AAC GAA CTG ACC GAC ACC CTG CAG  45
Gly Glu Phe Gly Pro Ser Gly Asn Glu Leu Thr Asp Thr Leu Gln
1    ↑               5                   10                  15
         L--- linker ------------┘ L------ fos-zipper ---------
GCT GAA ACC GAC CAG CTG GAA GAC AAA AAA TCC GCT CTG CAG ACC  90
Ala Glu Thr Asp Gln Leu Glu Asp Lys Lys Ser Ala Leu Gln Thr
                    20                  25                  30
-------------------- fos-zipper ----------------------------------
GAA ATC GCT AAC CTG CTG AAA GAA AAA GAA AAA CTG GAA TTT ATC  135
Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile
                    35                  40                  45
-------------------- fos-zipper ----------------------------------
CTG GCT GCT TAC TGATAAGCTT GACCTGTGAA GTGAAAAATGGCG          180
Leu Ala Ala Tyr          ↑
---------------┘ stop
```

3 3

5

Patent claims

1. Monomeric antibody-fragment fusion protein essentially consisting of a Fv-fragment of an antibody and a peptide
10   which is capable to dimerize with another peptide by noncovalent interaction.

2. Monomer according to claim 1 characterized in that the Fv-fragment is a single chain fragment.

15

3. Monomer according to claim 1 or 2 characterized in that the interactive peptide consists of 10 to 50, preferrably 10 to 30 amino acids.

20   4. Monomer according to one of the claims 1 or 3 characterized in that the peptide consists of at least one helix.

5. Monomer according to claim 4 characterized in that the helix peptide consists of a helix, a turn and another
25   helix.

6. Monomer according to claims 4 characterized in that the peptide contains a leucine zipper molecule, having several repeating amino acids, in which every seventh
30   amino acid is a leucine.

3 4

7. Monomer according to claims 4 characterized in that the
    peptide bears charged residues.

8. Monomer according one of the claims 1 to 7 characterized
5   in that a linking peptide is between the Fv-fragment and
    the peptide.

9. Monomer according to claim 8 characterized in that the
    linking peptide is a hinge region sequence of an antibody
10 or a fragment thereof.

10. Process for preparation of a monomeric antibody fusion
    protein as defined in claims 1 to 9, characterized in
    that the genes coding for the Fv-fragment, the interac-
15 tive peptide and, if desired, the linking peptide are
    cloned into one expression plasmid, a host cell is trans-
    formed with said expression plasmid and cultivated in a
    nutrient solution, and the monomeric fusion protein is
    expressed in the cell or secreted into the medium.
20
11. Process according to claim 10 characterized in that the
    host cell is E. coli.

12. Dimeric fusion protein essentially consisting of two
25 monomeric fusion proteins, wherein the linkage of the
    monomeric units bases on noncovalent interaction of
    identical or different peptides, characterized in that at
    least one monomeric unit is an antibody-fragment fusion
    protein as defined in claims 1 to 9.
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13. Dimeric fusion protein according to claim 12 wherein the interactive peptides are the same.

14. Dimer according to claim 12 or 13 characterized in that the second monomeric unit is an antibody-fragment fusion protein as defined in claims 1 to 9 having different specificity.

15. Dimer according to claim 12 or 13 characterized in that the second monomeric unit is a fusion protein as defined in claims 1 to 9, wherein the antibody-fragment (Fv) is replaced by a non-antibody protein or peptide.

16. Dimer according to claim 15 characterized in that the protein or peptide is a toxin, a chelator peptide, a metal binding protein or an enzyme, or has the corresponding specific binding site.

17. Dimer according to claim 15 characterized in that the protein or peptide has a T-cell-, or a T-cell fragment specific binding site.

18. Dimer according to one of the claims 12 to 17, wherein another protein is fused at the C-terminus of one or both of the intercalating peptides.

19. Dimer according to claim 18, wherein the fused protein is a toxin, a chelator peptide, a metal binding protein or an enzyme, or has the corresponding specific binding site, or has a T-cell (fragment) specific binding site.
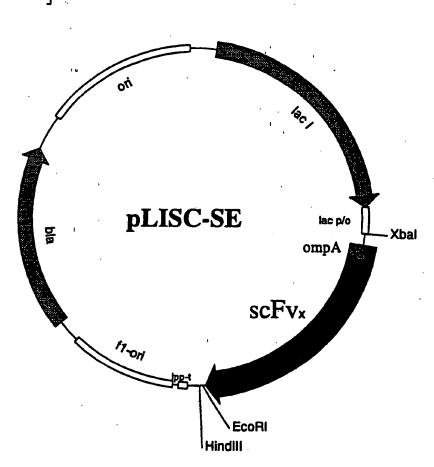
20. Process for preparation of a dimeric fusion proteinas
defined in claims 12 to 19 characterized in that the
genes coding for the complete monomeric fusion proteins
or parts of it are cloned at least into one expression

5   plasmid, a host cell is transformed with said expression
plasmid(s) and cultivated in a nutrient solution, and
either the complete dimeric fusion protein is expressed
in the cell or into the medium, or the monomeric fusion
proteins are separately expressed and the noncovalent

10   linkage between the two monomeric units is performed in
the medium or in vitro, and in the case that only parts
of the fusion proteins were cloned protein engineering
steps are additionally performed.

15 21. Process according to claim 20 characterized in that the
gene coding for the first monomeric fusion protein is
cloned into a first expression plasmid, and the gene
coding for the second monomeric fusion protein is cloned
into a second expression plasmid.

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22. Process according to claim 20 characterized in that the
noncovalent linkage between the monomeric units forming
the dimeric fusion protein is performed in vitro.

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23. Process according to one of the claims 20 to 22 characterized in that the host cell is E. coli.

24. Construction kit for preparation of seletive dimers of
5   antibody-fragment fusion proteins as defined in claims 12
       to 19 containing (a) a monomeric antibody-fragment fusion
       protein as defined in claims 1 to 8, and (b) a second
       monomeric fusion protein as defined in (a), wherein the
       antibody fragment has the same or another antigen speci-
10   ficity, or wherein the antibody fragment unit is replaced
       by a non-antibody protein/peptide.
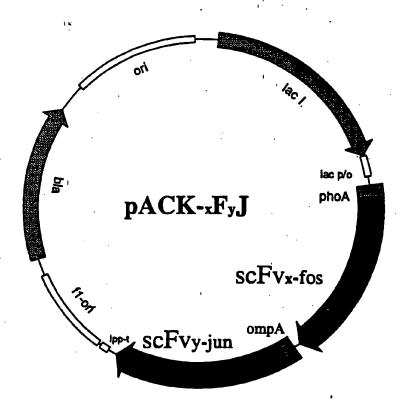

15

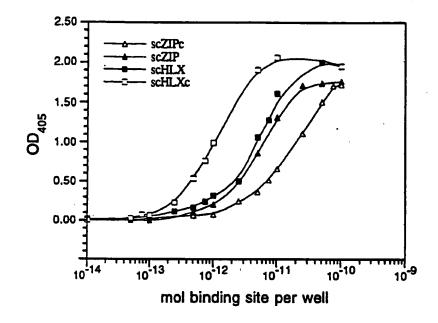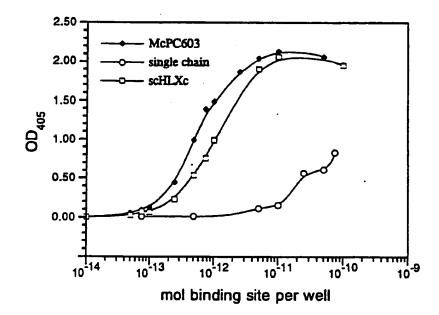20

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FIG. 1

FIG. 2

Fig. 3 a

Fig. 3 b

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No | PCT/EP 93/00082

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/62;   C12N15/13;   C07K15/28

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	TRENDS IN BIOTECHNOLOGY vol. 9, April 1991, CAMBRIDGE GB pages 132 - 137 BIRD AND WALKER 'Single chain antibody variable regions' see page 136, column 3, paragraph 2 ---	1-7, 10-24
Y	PROTEIN ENGINEERING vol. 4, no. 4, April 1991, ENGLAND GB pages 457 - 461 BLONDEL AND BEDOUELLE 'Engineering the quarternary structure of an exported protein with a leucine zipper' see page 461, column 1, last paragraph ---	1-7, 10-24
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 JUNE 1993	2 1 -06- 1993

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	CUPIDO M.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOCHEMISTRY vol. 31, no. 6, 18 February 1992, EASTON, PA US pages 1579 - 1584 PACK AND PLÜCKTHUN 'Miniantibodies: Use of amphipatic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in Escherichia coli' see the whole document	1-24